

1           **A Multiplex Real-Time PCR with High Resolution Melting Analysis for the**  
2           **Characterization of Antimicrobial Resistance in *Neisseria gonorrhoeae***

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25 **ABSTRACT**

26 Resistance to antibiotics used against *Neisseria gonorrhoeae* infections is a major public  
27 health concern. Antimicrobial resistance (AMR) testing relies on time-consuming culture-  
28 based methods. Development of rapid molecular tests for detecting AMR determinants could  
29 provide valuable tools for surveillance, epidemiological studies and to inform individual case  
30 management. We developed a fast (<1.5 hrs) SYBR-green based real-time PCR method with  
31 high resolution melting (HRM) analysis. One triplex and three duplex reactions included two  
32 sequences for *N. gonorrhoeae* identification and seven determinants of resistance to extended-  
33 spectrum cephalosporins (ESCs), azithromycin, ciprofloxacin, and spectinomycin. The  
34 method was validated by testing 39 previously fully-characterized *N. gonorrhoeae* strains, 19  
35 commensal *Neisseria* spp., and an additional panel of 193 gonococcal isolates. Results were  
36 compared with culture-based AMR determination. The assay correctly identified *N.*  
37 *gonorrhoeae* and the presence or absence of the seven AMR determinants. There was some  
38 cross-reactivity with non-gonococcal *Neisseria* species and the detection limit was  $10^3$ - $10^4$   
39 gDNA copies/reaction. Overall, the platform accurately detected resistance to ciprofloxacin  
40 (sensitivity and specificity, 100%), ceftriaxone (sensitivity 100%, specificity 90%), cefixime  
41 (sensitivity 92%, specificity 94%), azithromycin and spectinomycin (both sensitivity and  
42 specificity, 100%). In conclusion, our methodology accurately detects mutations generating  
43 resistance to antibiotics used to treat gonorrhea. Low assay sensitivity prevents direct  
44 diagnostic testing of clinical specimens but this method can be used to screen collections of  
45 gonococcal isolates for AMR more quickly than with current culture-based AMR testing.

46

## 47 INTRODUCTION

48 Gonorrhea is the second most common bacterial sexually transmitted infection worldwide,  
49 with an estimated 78 million new cases in 2012 (1). Moreover, *Neisseria gonorrhoeae* has  
50 developed resistance to most current and past treatment options. Antimicrobial resistant  
51 (AMR) gonorrhea is a major public health concern about which the World Health  
52 Organization (WHO) emphasizes the importance of global surveillance to identify emerging  
53 resistance, monitor trends, and inform revisions of treatment guidelines (2, 3).

54 At a molecular level, the mechanisms which confer resistance to the most common  
55 treatment options have been well characterized. For instance, the acquisition of mosaic *penA*  
56 alleles, with or without substitutions at amino acid position 501 of the encoded penicillin-  
57 binding protein 2 (PBP2), has been linked to decreased susceptibility or resistance to the  
58 extended-spectrum cephalosporins (ESCs) cefixime (CFX) and ceftriaxone (CRO) (4, 5). In  
59 particular, strains harboring a mosaic XXXIV *penA* gene, including the internationally-  
60 spreading *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) genogroup 1407, have  
61 been responsible for ESC treatment failures in several countries worldwide (5-8). The  
62 mutations A2059G or C2611T in the 23S rRNA alleles are associated with resistance to  
63 azithromycin (AZM) (9, 10), whereas a Ser91Phe substitution in GyrA results in  
64 ciprofloxacin (CIP) non-susceptibility (11). Single nucleotide polymorphisms (SNPs) in the  
65 16S rRNA or in the ribosomal protein S5 (RPS5) encoding gene *rpsE* (12, 13) confer  
66 spectinomycin (SPC) resistance. However, we should note that while the CIP-resistant *N.*  
67 *gonorrhoeae* isolates are frequently observed, those fully resistant to ESCs, AZM and SPC  
68 are still sporadically found (14, 15).

69 Nucleic acid amplification testing (NAAT) has already replaced culture-based  
70 detection of *N. gonorrhoeae* in many settings, but these methods do not provide any  
71 information about AMR (16). On the other hand, antimicrobial susceptibility testing (AST) is  
72 usually performed with time-consuming culture methods (16). For this reason, there has been

73 growing interest in the development of NAATs that can supplement culture-based AMR  
74 testing, enhance AMR surveillance and, ideally, be used to tailor individualized treatment for  
75 gonorrhea patients (17).

76         Several nucleic acid amplification-based methods have been developed to identify the  
77 presence of SNPs (18). One of these techniques is high resolution melting (HRM) analysis,  
78 which relies on the detection of changes in the melting temperature ( $T_m$ ) resulting from the  
79 presence of mutations in a previously amplified target. This method is so sensitive that even  
80  $T_m$  shifts derived from one SNP can be detected (19). Moreover, strategic target design (i.e.,  
81 distinct  $T_m$  of the amplicons) also allows multiplexing of more than one reaction per single  
82 tube (20). However, only multiple-step (e.g., requirement of additional steps after nucleic acid  
83 amplification for read-out) (21, 22), or single-antibiotic (e.g., only resistance to CIP or only  
84 to AZM) NAAT-based methodologies to characterize AMR gonorrhea have been proposed in  
85 the past (23-28).

86         In this study, we developed and evaluated a new SYBR-green based real-time PCR  
87 method with HRM analysis to simultaneously detect *N. gonorrhoeae* and key mutations  
88 associated with ESCs, AZM, CIP and SPC resistance in four closed-tube multiplex reactions.

## 89 MATERIALS AND METHODS

90 **Design of the real-time PCR assay.** Nine primer sets were designed with the Oligo Primer  
91 Analysis software v4.0 (Molecular Biology Insights) to amplify specific sequences of the  
92 targets described in Table 1. Primers were designed to flank the mutation site of interest in  
93 *gyrA*, 23S rRNA, 16S rRNA and *rpsE* genes, and to amplify *penA* mosaic sequences (e.g.,  
94 pattern XXXIV) around codons 501 and 545. Additionally, GC clamps were added at the 5'-  
95 end of some oligonucleotides to shift the  $T_m$  of the resulting amplicons in order to separate  
96 the peaks for easier interpretation of multiplex reactions. The nine primer sets generated ~40-  
97 140 bp products and all operated at the same conditions both in single- and multiplex  
98 reactions (Table 1).

99 *N. gonorrhoeae* isolates were grown on GC agar (bioMérieux) for 24 hrs at 35°C in a  
100 humid 5% CO<sub>2</sub>-enriched atmosphere. Genomic DNA extraction was performed using the  
101 QIAamp DNA mini kit (QIAGEN). Each 20 µl reaction contained 0.3 µM of each primer, 1X  
102 Meltdoctor Master Mix (Applied Biosystems), and 20 ng of genomic DNA (gDNA).  
103 Experiments were run on a QuantStudio 7 Flex instrument (Applied Biosystems). The PCR  
104 stage included a first denaturation step (95°C, 10 min), followed by 30 cycles of denaturation  
105 (95°C, 15 sec), annealing (62°C, 10 sec), and extension (72°C, 10 sec). After amplification,  
106 HRM analysis was performed using the following parameters: after 10 sec at 95°C and a 60°C  
107 hold for 1 min, the fluorescence signal was collected, while the samples were heated up from  
108 60°C to 95°C with a ramping time of 0.025°C/sec. Results were analyzed with the  
109 QuantStudio 6 and 7 Flex Real-Time PCR Software v1.0 (Applied Biosystems). Overall,  
110 starting from extracted DNA templates the results were available in <1.5 hrs (i.e., real-time  
111 PCR amplification of <60 min followed by HRM analysis of <30 min). To assess the limit of  
112 detection (LOD) of our molecular method, known quantities of gDNA copies/reaction were  
113 tested in ten-fold serial dilutions.

114 *Neisseria spp. control strains.* A panel of 35 *N. gonorrhoeae* isolates was used to validate the  
115 real-time PCR method. The panel included: 26 previously fully-characterized isolates with  
116 known profiles of MICs and genetic resistance determinants (14); the fully sensitive reference  
117 strain ATCC 49226; WHO reference strains WHO K (carrying a mosaic X *penA* gene),  
118 WHO L, WHO P, the SPC-resistant WHO O (with the 16S rRNA C1192T substitution; MIC  
119 >1024 µg/ml) and WHO A (with the RPS5 Thr24Pro substitution; MIC, 128 µg/ml) (29);  
120 two AZM-resistant strains, AZM-HLR (harboring four 23S rRNA alleles with the A2059G  
121 mutation; MIC ≥256 µg/ml) and G07 (harboring four 23S rRNA alleles with the C2611T  
122 mutation; MIC, 8 µg/ml); and the ESC-resistant strain F89 carrying a mosaic XXXIV *penA*  
123 gene with an additional mutation in codon 501 leading to an Ala501Pro substitution (MICs  
124 for CFX and CRO of 2 and 1.5 µg/ml, respectively) (5).

125 Nineteen non-gonococcal *Neisseria spp.* strains previously identified with the matrix-assisted  
126 laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS, Bruker  
127 Daltonik) were also used to assess cross-reactivity. The panel included: *N. meningitidis* (n=5),  
128 *N. mucosa* (n=3), *N. sicca* (n=2), *N. cinerea* (n=2), *N. lactamica* (n=2), *N. subflava* (n=1), *N.*  
129 *flava* (n=1), *N. flavescens* (n=1), *N. elongata* (n=1), and *N. bacilliformis* (n=1).

130 ***Analysis of representative spiked negative and positive samples.*** Pharyngeal, rectal and  
131 urethral clinical specimens were collected with ESwabs (Copan) and tested for *N.*  
132 *gonorrhoeae* by APTIMA Combo 2 (Hologic). The QIAamp DNA Mini kit (Qiagen) was  
133 used to extract total DNA from 200 µl of ESwabs with positive or negative APTIMA results.  
134 For the assessment of negative spiked specimens, 2 µl of sample DNA obtained from ESwab  
135 were spiked with additional 10<sup>5</sup>, 10<sup>4</sup> or 10<sup>3</sup> gDNA copies of the appropriate control *N.*  
136 *gonorrhoeae* strain per reaction for each multiplex. For the positive specimens, 2 µl of sample  
137 DNA were used for each multiplex reaction. Culture isolates from the specimens were  
138 obtained with standard microbiological methods and species identification (ID) was achieved  
139 using the MALDI-TOF MS.

140 **Analysis of gonococcal isolates and statistical analysis.** We analyzed 193 *N. gonorrhoeae*  
141 isolates collected during a 25-year period (1989-2014) in two microbiology laboratories  
142 located in Switzerland (Institute for Infectious Diseases, University of Bern, Bern; Institute of  
143 Medical Microbiology, University Hospital Zürich, Zürich) with both culture-based AST and  
144 the new real-time PCR method.

145 ID was achieved using the MALDI-TOF MS. MICs for CFX, CRO, CIP, AZM and SPC were  
146 obtained on GC agar plates (bioMérieux) (30) using the Etest method. MIC values for CFX,  
147 CRO, CIP and SPC were categorized using the 2015 European Committee on Antimicrobial  
148 Susceptibility Testing (EUCAST) criteria (31). For AZM, we defined moderate- and high-  
149 level resistance as MICs >2 to 128 and  $\geq 256$   $\mu\text{g/ml}$ , respectively, as previously published (9).

150 Positive results from the real-time PCR assay (based on both amplification and  
151 melting temperature analysis) were interpreted as follow: *i)* *opa* and/or *porA*, strain identified  
152 as *N. gonorrhoeae*; *ii)* *penA* encoding for Gly545Ser substitution and/or *penA* Ala501, strain  
153 resistant to CFX and/or CRO; *iii)* 23S rRNA C2611T or A2059G mutations, strain  
154 moderately or highly resistant to AZM, respectively; *iv)* *gyrA* encoding for Ser91Phe  
155 substitution, strain non-susceptible to CIP; and *v)* *rpsE* encoding for Thr24Pro substitution or  
156 16S rRNA C1192T mutation, strain resistant to SPC. Each sample was run in duplicate. Due  
157 to small inter-assay variabilities of the  $T_m$  (Table 2), positive controls for each reaction (e.g.,  
158 harboring the mutated AMR target sequence) were included to facilitate the interpretation of  
159 the results. Inconsistent results were confirmed by repetition of the real-time PCR and  
160 PCR/DNA sequencing.

161 For the 193 isolates, we calculated the sensitivity (with 95% CI) of the real-time PCR  
162 with HRM analysis for the detection of *N. gonorrhoeae* compared with MALDI-TOF MS  
163 used as the reference standard. We calculated sensitivity (with 95% CI) for the detection of  
164 AMR to each antibiotic class as the percentage of isolates with a non-susceptible or resistant  
165 MIC value that were correctly identified by a positive HRM result for the presence of the

166 correlated resistance determinant. We calculated specificity (with 95% CI) as the percentage  
167 of isolates with a susceptible MIC value that were correctly identified by a negative HRM  
168 result for the correlated resistance determinant.

169 Since the 193 isolates detected in Switzerland did not include the rare strains possessing the  
170 mutations conferring fully resistance to CRO, AZM and SPC, sensitivity and specificity were  
171 also calculated including the results for the 35 *N. gonorrhoeae* control strains and four  
172 additional isolates provided by the WHO Collaborating Centre for Gonorrhoea and other STIs  
173 (Örebro, Sweden). Those four included: the ESC-resistant strain A8806 harboring a mosaic  
174 *penA* allele (MICs for CFX and CRO of 2 and 0.5 µg/ml, respectively) (32); the AZM-  
175 resistant strains GC2 (33) and GC4 harboring the C2611T (AZM MIC of 8 µg/ml) and  
176 A2059G (AZM MIC of  $\geq 256$  µg/ml) mutation in all four 23S rRNA alleles, respectively; and  
177 the SPC-resistant strain GC3 harboring the 16S rRNA C1192T mutation (MIC for SPC of  
178  $>1024$  µg/ml).



179 **RESULTS AND DISCUSSION**

180 One triplex and three duplex reactions were designed to characterize target sequences specific  
181 for *N. gonorrhoeae* identification (*opa* and *porA*) (34, 35), as well as for resistance to ESCs  
182 (mosaic *penA* alleles), CIP (GyrA substitution), AZM (23S rRNA mutations), and SPC (16S  
183 rRNA mutation or RPS5 substitution) (Table 1).

184 **Validation of the method and limit of detection (LOD).** As shown in Table 2, all 35 *N.*  
185 *gonorrhoeae* control strains were correctly identified by the positive amplification of both *opa*  
186 and *porA* reactions; amplicons had an average  $T_m$  of 76.98°C and 74.36°C, respectively, by  
187 HRM analysis.

188 The *penA* reaction targeting Gly545Ser was relatively specific for mosaic *penA* patterns. Only  
189 non-mosaic pattern XIX was cross-amplified, but all *N. gonorrhoeae* strains harboring a  
190 mosaic *penA* allele (i.e., pattern XXXIV and X) were correctly identified by the presence of  
191 the Gly545Ser, which caused a mean  $T_m$  shift of 0.46°C compared with the wild-type  
192 sequence. Additionally, the Ala501 reaction only amplified mosaic *penA* patterns, but we  
193 were not able to detect the mutation encoding the Ala501Pro substitution found in the ESC-  
194 resistant F89 strain (Table 2) (5). This was probably because third class mutations (i.e., G to C  
195 SNPs) are known to be difficult to detect by HRM, since the  $T_m$  shift resulting from such  
196 nucleotide substitutions is very small (15). Nevertheless, we kept this reaction for  
197 confirmation of the presence of mosaic *penA* alleles.

198 HRM analysis correctly identified the presence or absence of mutations associated with  
199 resistance to ciprofloxacin, azithromycin and spectinomycin (Table 2). Strains harboring the  
200 Ser91Phe substitution in GyrA generated discernible melting curves compared with the wild-  
201 type isolates with a mean  $T_m$  difference ( $\Delta T_m$ ) of 0.61°C. One strain (2121127) (14),  
202 harbored an additional mutation in codon 92, which caused a further shift in the  $T_m$  when  
203 compared with the wild-type sequence ( $\Delta T_m$ = 1.25°C). Strains with mutations A2059G or  
204 C2611T in all four alleles of the 23S rRNA generated unique profiles compared with isolates

205 harboring wild-type alleles, with mean  $\Delta T_m$  of 0.22°C and 0.75°C, respectively. Strains  
206 harboring the target SNPs in *rpsE* or 16S rRNA exhibited a mean  $T_m$  shift of 0.68-0.69°C  
207 compared with the wild-type sequences (Table 2).

208 Finally, when testing 10-fold dilutions of  $10^7$  to 10 gonococcal gDNA copies/reaction, a  
209 starting quantity of at least  $10^3$ - $10^4$  gDNA copies was needed to allow proper HRM analysis  
210 in all four multiplex reactions (see examples in Figure S1). This is higher than available  
211 commercial platforms (e.g., according to the manufacturer, the APTIMA Combo2 test claims  
212 an analytical sensitivity of 50 cells/assay).

213 **Cross-reaction with non-gonococcal *Neisseria* spp.** The production of false-positive results  
214 due to the presence of non-gonococcal *Neisseria* spp. commonly found in some specimen  
215 types (e.g., pharyngeal and rectal samples) is a major challenge for the design of NAAT-  
216 based diagnostic methods. In fact, several *Neisseria* spp. share with the gonococcus a high  
217 sequence similarity for some of the targets (e.g., 23S rRNA and 16S rRNA genes). Moreover,  
218 the *N. gonorrhoeae* mosaic *penA* allele is thought to be the result of horizontal gene transfer  
219 of the commensal orthologues (36, 37). Therefore, in order to assess the level of cross-  
220 reactivity for all nine genetic targets included in our multiplex real-time PCR platform, a  
221 panel of ten different non-gonococcal *Neisseria* species (overall, 19 strains) was tested.

222 As shown in Table S1, none of these strains showed positive amplification for *opa* and  
223 *porA*. This was expected, since both genetic regions were previously proven to be specific for  
224 *N. gonorrhoeae* (34, 35). The GyrA Ser91Phe reaction was also specific for *N. gonorrhoeae*.  
225 In contrast, several non-gonococcal species showed cross-reactions for all remaining target  
226 sequences (Table S1). In only a few cases, cross-amplification could be distinguished from *N.*  
227 *gonorrhoeae* by a different  $T_m$  (i.e., 23S rRNA A2059G), but for most targets the  $T_m$  of the  
228 amplified commensal target matched the expected  $T_m$  of the gonococcal wild-type sequence  
229 (e.g., 23S rRNA C2611, 16S rRNA C1192). However, none of the cross-reacting species had  
230 a  $T_m$  equal to that of the mutated *N. gonorrhoeae* sequence for any of the targets, indicating

231 that false-positives deriving from the presence of commensals are unlikely. Even in the  
232 presence of a positive *penA* A501 reaction, the lack of amplification of target sequence *penA*  
233 Gly545Ser or the absence of the Gly545Ser substitution allowed the differentiation of the  
234 gonococcal mosaic *penA* gene from its commensal counterpart, since this substitution is  
235 mostly found in gonococcus. On the other hand, excessive amounts of wild-type amplification  
236 due to commensal *Neisseria* spp. could potentially mask the presence of an AMR mutation in  
237 *N. gonorrhoeae*, especially in clinical specimens with low load of the pathogen (i.e., in  
238 pharyngeal samples) (38, 39).

239 ***Analysis of the representative spiked negative and positive samples.*** To assess the extent of  
240 commensal interference on the detection of the AMR determinants in clinical specimens, four  
241 pharyngeal and four rectal samples negative for *N. gonorrhoeae* were spiked with gDNA of  
242 control strains possessing the mutations of interest for each multiplex reaction.

243 The results obtained from the pharyngeal specimens showed strong background amplification  
244 of wild-type amplicons due to the presence of *Neisseria* spp. for most target reactions (e.g.,  
245 23S rRNA C2611T, 16S rRNA C1192T, *rpsE* Thr24Pro). This background amplification  
246 would cause false negative results especially in the presence of low amounts of gonococcus.  
247 Additionally, nonspecific amplification strongly affected the melting curve interpretation of  
248 the *gyrA* Ser91Phe and 23S rRNA A2059G reactions. Finally, two samples exhibited positive  
249 amplification of the *penA* A501 reaction due to commensals (see examples in Figure S2 A-E).  
250 On the other hand, for the spiked negative rectal specimens, only strong cross-amplification  
251 of wild-type 16S rRNA C1192 was observed (see examples in Figure S3 A-D).

252 Taken together with the relatively high LOD needed for proper HRM analysis, these  
253 limitations suggested that our method would not be suitable for direct screening of clinical  
254 specimens. For this reason, total DNA extracted from four pharyngeal, four rectal and four  
255 urethral clinical samples positive for *N. gonorrhoeae* was used to test the performance of our

method. Results were also compared to the gDNA extracted from *N. gonorrhoeae* strains (when available) isolated from the specimens.

Our platform indicated that all four pharyngeal samples tested positive for the *opa* reaction (Figure S4 A-D). Cross-amplification of commensals together with the relatively low gonococcal load led to a false positive result for the presence of a mosaic *penA* in one sample. Additionally, the melting curves of several reactions were not properly interpretable due to low or nonspecific amplification (e.g., *gyrA* Ser91Phe, 23S rRNA A2059G, *rspE* Thr24Pro). Similarly, low amplicon amounts strongly affected the melting curve interpretation of all four multiplex reactions in the positive rectal (Figure S5 A-D) and urethral specimens (Figure S6 A-D), confirming that our method cannot be directly implemented for clinical specimens. Nonetheless, it could be a valuable tool for rapid screening of large isolate collections, both for surveillance and epidemiological purposes. For this reason, we compared our molecular methodology with the standard culture-based AST Etest method for a panel of 193 Swiss isolates.

**Analysis of the 193 clinical isolates.** As shown in Table 3, the real-time PCR platform correctly identified all isolates as *N. gonorrhoeae*. Moreover, AMR characterization for CIP had both sensitivity and specificity of 100%, whereas AZM and SPC had specificity of 100%. In particular, our method correctly identified all isolates exhibiting resistance to CIP (58 out of 58). No mutations associated to SPC resistance were observed in agreement with the results obtained by phenotypic AST. Furthermore, none of the isolates tested positive for the 23S rRNA C2611T or A2059G mutations associated with moderate or high AZM resistance, respectively. Consistently, none of the tested isolates exhibited AZM MICs >2 µg/ml. Finally, all 7 strains showing CFX resistance by phenotypic AST were positive for the presence of a mosaic *penA* allele. However, no resistance to CRO was observed. This was expected, since it is known that the presence of a mosaic *penA* gene is typically associated to raised MICs for ESCs, even if usually still in the susceptible range based on EUCAST criteria (40).

282 Thus, we further explored the MIC distribution of CFX and CRO in isolates harboring mosaic  
283 or non-mosaic *penA* patterns (Figure 1). Out of the 16 isolates positive for the presence of a  
284 mosaic *penA* allele, seven were CFX resistant and five were only a two-fold dilution apart  
285 from being resistant (MIC, 0.125 µg/ml). The remaining four strains with a mosaic *penA* gene  
286 had raised CFX MICs of 0.064-0.094 µg/ml, whereas all other non-mosaic isolates tested  
287 exhibited MICs of ≤0.047 µg/ml. Furthermore, all 16 strains harboring a mosaic *penA* allele  
288 also showed raised CRO MICs in the range of 0.023 to 0.094 µg/ml, which were noticeably  
289 higher compared to strains with non-mosaic patterns, in agreement with previous observations  
290 (37, 40, 41).

291 **Overall performance of the real-time PCR platform.** Since some of the resistance mutations  
292 were not included among the 193 Swiss isolates, we also evaluated the performance of our  
293 test including the 35 control strains and 4 additional isolates harboring known, but very rare,  
294 AMR determinants (Table 3).

295 Our platform accurately identified *N. gonorrhoeae* with a sensitivity and specificity of 100%.  
296 However, strain GC2 tested positive only for the *opa* reaction. Notably, this strain was  
297 previously reported to cause false-negative results in other *porA*-based PCRs due to the  
298 acquisition of a meningococcal *porA* allele (33). For this reason, our dual-target approach  
299 proved to be extremely valuable for the identification of even such exceptional isolates.

300 With regard to the AMR detection, the platform correctly predicted resistance to ciprofloxacin  
301 in all 83 strains positive for a mutation in codon 91 of *gyrA*. Furthermore, the prediction of a  
302 mosaic *penA* allele allowed the detection of two fully CRO-resistant strains (F89 and A8806),  
303 as well as all isolates resistant to CFX with the exception of WHO L, which harbors a non-  
304 mosaic *penA* allele with an additional substitution in amino acid 501. It is worth noting that  
305 the mosaic *penA* allele of A8806 differs from the pattern XXXIV allele found in the high-  
306 level CRO-resistant F89 strain. For this reason, no amplification of the *penA* Gly545Ser target  
307 was observed for A8806. Nevertheless, the strain was correctly identified as harboring a

308 mosaic *penA* allele due to the positive *penA* Ala501 reaction. Finally, the identification of  
309 either of the two mutations conferring resistance to AZM or SPC was correctly associated  
310 with resistance to those antibiotics.

311 **Conclusions.** We developed and validated a new real-time PCR method coupled with HRM  
312 analysis that accurately detected several important mutations associated with resistance to  
313 antibiotics commonly used to treat gonorrhea. Cross-reactivity with commensal species and  
314 high limit of detection suggested that our method is not suitable for direct screening of  
315 clinical specimens. However, it proved to be a useful and rapid alternative to culture-based  
316 methods to assess the AMR profiles for ESCs, AZM, CIP and SPC of a large collection of *N.*  
317 *gonorrhoeae* isolates.

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459 **TABLE 1.** Target genes, primer sequences, amplicon lengths, mutations and affected antibiotics, and multiplex combinations of the real-time PCR platform

Target, mutation	Primer name and oligonucleotide sequences <sup>a</sup>	Amplicon length	Associated target and antibiotic affected	Multiplex
<i>opa</i>	<i>opa</i> _F 5'-gttcatccgccatattgttga-3'	56	<i>opa</i> (Species identification)	Triplex
	<i>opa</i> _R 5'-aaggcgcgattatateggttcc-3'			
<i>porA</i>	<i>porA</i> _F 5'-cagcaattgttccgagtc-3'	44	<i>porA</i> (Species identification)	Triplex
	<i>porA</i> _R 5'-ggcggtatagcggaactg-3'			
<i>penA</i> Gly545Ser	545_F 5'-ccgccccgccgactgcaaacggttacta-3'	61	Mosaic <i>penA</i> (Decreased susceptibility/resistance to ESCs)	Triplex
	545_R 5'-ccgccccgccgacctgccaactacac-3'			
<i>penA</i> Ala501	501_F 5'-ccgccccgccgctggcgcaaaacggtagc-3'	79	Mosaic <i>penA</i> (Decreased susceptibility/resistance to ESCs)	Duplex I
	501_R 5'-ccgccccgccaatcgacgtaacgacctgaaccaacttacg-3'			
23S rRNA C2611T	C2611_F 5'-acgtcgtgagacagtttggtc-3'	49	23S rRNA C2611T (Moderate AZM resistance) <sup>b</sup>	Duplex I
	C2611_R 5'-caaactccaacgccactgc-3'			
23S rRNA A2059G	A2059_F 5'-ctacccgctgctagacgga-3'	142	23S rRNA A2059G (High AZM resistance) <sup>b</sup>	Duplex II
	A2059_R 5'-caggggtggtatttcaaggacga-3'			
<i>gyrA</i> Ser91Phe	<i>gyrA</i> _S91_F 5'-taaataccacccccacggcgatt-3'	47	<i>GyrA</i> Ser91Phe (CIP resistance)	Duplex II
	<i>gyrA</i> _S91_R 5'-atacggacgatggtgtcgtaaact-3'			
<i>rpsE</i> Thr24Pro	S5_T24_F 5'-atggtcgcagttaccgtgta-3'	56	RPS5 Thr24Pro (SPC resistance)	Duplex III
	S5_T24_R 5'-aaagccataatgcaccac-3'			
16S rRNA C1192T	16S_1192_F 5'-cgcccccgagggaaggtgggatga-3'	64	16S rRNA C1192T (SPC resistance)	Duplex III
	16S_1192_R 5'-cgccccctggtcataaggccatgag-3'			

460 **Note.** ESCs, extended spectrum cephalosporins; AZM, azithromycin; CIP, ciprofloxacin; SPC, spectinomycin461 <sup>a</sup> GC clamps, which were added to the 5'-end of some primers to allow multiplexing, are shown in italics.462 <sup>b</sup> They confer moderate- to high-level resistance to AZM (i.e., MIC > 2 µg/ml) when at least 3 out of 4 copies are mutated (9)

463

464 **TABLE 2.** Results of the method validation using the 35 well-characterized *N. gonorrhoeae* isolates

Target sequence	Sequence type of the 35 control isolates by:		T <sub>m</sub> (°C)		Mean ΔT <sub>m</sub> ± SD (°C)	Sensitivity, <sup>c</sup> % (95% CI)	Specificity, <sup>c</sup> % (95% CI)
	DNA sequencing	Real-time PCR/HRM analysis	Range	Mean ± SD			
<i>opa</i>	Positive (n=35)	Positive (n=35)	76.63 - 77.22	76.98 ± 0.13	n/a	100 (90-100)	n/a <sup>d</sup>
	Negative (n=0)	Negative (n=0)	n/a	n/a	n/a	100 (90-100)	n/a <sup>d</sup>
<i>porA</i>	Positive (n=35)	Positive (n=35)	73.79 - 74.88	74.36 ± 0.20	n/a	100 (90-100)	n/a <sup>d</sup>
	Negative (n=0)	Negative (n=0)	n/a	n/a	n/a	100 (90-100)	n/a <sup>d</sup>
<i>penA</i> Gly545Ser	Non-mosaic (n=23)	Non-mosaic (n=23)	n/a <sup>a</sup>	n/a <sup>a</sup>			
	Non-mosaic Gly545 (ggc) (n=3)	Non-mosaic Gly545 (ggc) (n=3)	85.05 - 85.23 <sup>a</sup>	85.14 ± 0.08 <sup>a</sup>	0.46 ± 0.05	100 (66-100)	100 (87-100)
	Mosaic Gly545Ser (agc) (n=9)	Mosaic Gly545Ser (agc) (n=9)	84.09 - 84.72	84.47 ± 0.20			
<i>penA</i> Ala501	Non-mosaic (n=26)	Non-mosaic (n=26)	n/a <sup>b</sup>	n/a <sup>b</sup>		100 (66-100)	100 (87-100)
	Mosaic (n=9)	Mosaic (n=9)	83.59 - 84.35	84.17 ± 0.19	n/i		
<i>gyrA</i> Ser91Phe	GyrA Ser91 (tcc), Ala92 (gca) (n=11)	GyrA Ser91 (tcc), Ala92 (gca) (n=11)	77.97 - 78.16	78.08 ± 0.05		100 (86-100)	100 (72-100)
	GyrA Ser91Phe (ttc), Ala92 (gca) (n=23)	GyrA Ser91Phe (ttc), Ala92 (gca) (n=23)	77.29 - 77.59	77.47 ± 0.07	0.61 ± 0.06		
	GyrA Ser91Phe (ttc), Ala92Ser (tca) (n=1)	GyrA Ser91Phe (ttc), Ala92Ser (tca) (n=1)	76.15 - 76.17	76.16 ± 0.02	1.25 ± 0.01		
23S rRNA A2059G	A2059 (n=34)	A2059 (n=34)	81.33 - 81.52	81.44 ± 0.03	0.22 ± 0.02	100 (3-100)	100 (90-100)
	A2059G (n=1)	A2059G (n=1)	81.61 - 81.70	81.67 ± 0.03			
23S rRNA C2611T	C2611 (n=34)	C2611 (n=34)	75.69 - 76.33	76.12 ± 0.16	0.75 ± 0.05	100 (3-100)	100 (90-100)
	C2611T (n=1)	C2611T (n=1)	75.08 - 75.55	75.30 ± 0.20			
<i>rpsE</i> Thr24Pro	Thr24 (acc) (n=34)	Thr24 (acc) (n=34)	73.87 - 74.34	74.08 ± 0.07	0.68 ± 0.01	100 (3-100)	100 (90-100)
	Thr24Pro (ccc) (n=1)	Thr24Pro (ccc) (n=1)	74.66 - 74.94	74.76 ± 0.09			
16S rRNA C1192T	C1192 (n=34)	C1192 (n=34)	81.38 - 81.72	81.56 ± 0.08	0.69 ± 0.01	100 (3-100)	100 (90-100)
	C1192T (n=1)	C1192T (n=1)	80.74 - 80.94	80.82 ± 0.09			

Note. T<sub>m</sub>, melting temperature; ΔT<sub>m</sub>, melting temperature difference between wild-type and mutated sequence; n/a, not applicable; n/i, not interpretable

<sup>a</sup> Only non-mosaic pattern XIX (with *penA* Gly545) showed cross-amplification

<sup>b</sup> No amplification was observed for all other non-mosaic *penA* pattern tested

<sup>c</sup> Sensitivity is the probability that an isolate was correctly identified as positive by HRM analysis for the target sequence (species ID, mosaic or mutation); specificity was the probability that an isolate was correctly identified as negative by HRM analysis for the target sequence (species ID, mosaic or mutation).

<sup>d</sup> Specificity was 100% considering that all 19 non-gonococcal control strains were correctly characterized as non-*N. gonorrhoeae* (see Table S1)

71 **TABLE 3.** Performance of the real-time PCR platform in characterizing the collection of 193 *N. gonorrhoeae* isolates alone and combined with the 39 *N. gonorrhoeae* control strains

Phenotypic target	Target sequence	<i>N. gonorrhoeae</i> isolates collected during 1989-2014 (n=193)						Overall <i>N. gonorrhoeae</i> strains (n=232), including the 39 controls					
		Test result	No. of isolates <sup>a</sup>	AST <sup>b</sup>		Sensitivity <sup>d</sup> % (95% CI)	Specificity <sup>d</sup> % (95% CI)	Test result	No. of strains <sup>a,c</sup>	AST <sup>b</sup>		Sensitivity <sup>d</sup> % (95% CI)	Specificity <sup>d</sup> % (95% CI)
				S	R					S	R		
Species identification	<i>opa</i> and/or <i>porA</i>	Positive	193			100 (97-100)	n/a	Positive <sup>e</sup>	232			100 (98-100) <sup>e</sup>	100 (82-100) <sup>e</sup>
		Negative	-	n/a	n/a			Negative <sup>e</sup>	19	n/a	n/a		
Ceftriaxone (CRO)	<i>penA</i> Gly545Ser and/or <i>penA</i> Ala501	Positive	16	16	-	n/a	92 (87-95)	Positive	26	24	2	100 (16-100)	90 (85-93)
		Negative	177	177	-			Negative	206	206	-		
Cefixime (CFX)	<i>penA</i> Gly545Ser and/or <i>penA</i> Ala501	Positive	16	9	7	100 (47-100)	95 (91-98)	Positive	26	14	12	92 (64-100)	94 (90-96)
		Negative	177	177	-			Negative	206	205	1 <sup>f</sup>		
Azithromycin (AZM) <sup>g</sup>	23S rRNA A2059G or 23S rRNA C2611T	Positive	-	-	-	n/a	100 (97-100)	Positive	4	-	4	100 (40-100)	100 (98-100)
		Negative	193	193	-			Negative	228	228	-		
Ciprofloxacin (CIP)	<i>gyrA</i> Ser91Phe	Positive	58	-	58	100 (91-100)	100 (96-100)	Positive	83	-	83	100 (96-100)	100 (98-100)
		Negative	135	135	-			Negative	149	149	-		
Spectinomycin (SPC)	<i>rpsE</i> Thr24Pro or 16S rRNA C1192T	Positive	-	-	-	n/a	100 (97-100)	Positive	3	-	3	100 (29-100)	100 (98-100)
		Negative	193	193	-			Negative	229	229	-		

72 **Note.** AST, antimicrobial susceptibility testing obtained with Etest; R, resistant; S, susceptible; CI, confidence interval; -, zero; n/a, not applicable73 <sup>a</sup>Numbers are based on the results of the multiplex real-time PCR platform74 <sup>b</sup>AST was categorized based on EUCAST criteria with exception for AZM (see below)75 <sup>c</sup>AZM resistance were defined as > 2 µg/ml76 <sup>d</sup>Sensitivity was the probability that an isolate categorized as resistant was identified as positive by real-time PCR; specificity was the probability that an isolate categorized as sensitive was identified as negative by

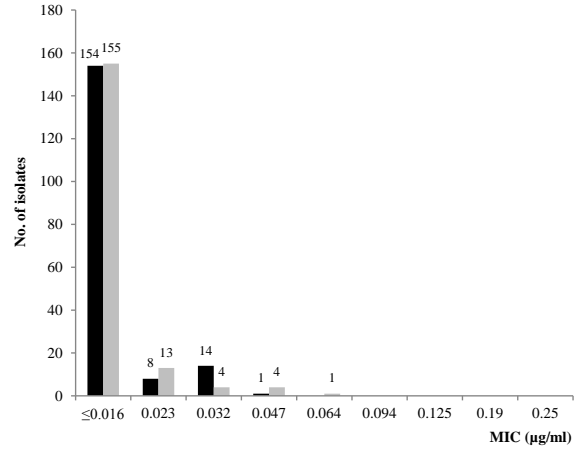
77 real-time PCR

78 <sup>e</sup>For the evaluation of the "Species identification" we also included the 19 non-gonococcal *Neisseria* spp. strains79 <sup>f</sup>Strain WHO L (non mosaic *penA* gene with an additional substitution in amino acid 501)

480 **LEGEND TO FIGURE 1**

481 Ceftriaxone (black bars) and cefixime (grey bars) MIC distribution of the 193 gonococcal  
482 isolates. **A**, isolates harboring a non-mosaic *penA* gene (n=177); **B**, isolates carrying a mosaic  
483 *penA* gene (n=16).

A.



B.

